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RESEARCH PAPER

# Vitamin D inhibits growth of human airway smooth muscle cells through growth factor-induced phosphorylation of retinoblastoma protein and checkpoint kinase 1

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**Background and purpose:** Airway remodelling in asthma is manifested, in part, as increased airway smooth muscle (ASM) mass, reflecting myocyte proliferation. We hypothesized that calcitriol, a secosteroidal vitamin D receptor (VDR) modulator, would inhibit growth factor-induced myocyte proliferation.

**Experimental approach:** Human ASM cell cultures were derived from bronchial samples taken during surgery. ASM cells were treated with platelet-derived growth factor (PDGF) (10 ng·mL$^{-1}$) for 24 h in the presence of calcitriol, dexamethasone or a checkpoint kinase 1 (Chk1) inhibitor (SB218078). The effects of calcitriol on PDGF-mediated cell proliferation were assessed by thymidine incorporation assay, propidium iodide-based cell cycle analysis, caspase-3 assay and immunoblotting for specific cell cycle modulators.

**Key results:** Calcitriol, but not dexamethasone, inhibited PDGF-induced ASM DNA synthesis concentration dependently ($IC_{50}$ = 520 ± 52 nM). These effects were associated with VDR-mediated expression of cytochrome CYP24A1 with no effects on ASM apoptosis. Calcitriol substantially inhibited ($P < 0.01$) PDGF-stimulated cell growth in ASM derived from both normal (59 ± 8%) and asthmatic subjects (57 ± 9%). Calcitriol inhibited PDGF-induced phosphorylation of retinoblastoma protein (Rb) and Chk1, with no effects on PDGF-mediated activation of extracellular signal-regulated kinases 1/2, PI3-kinase and S6 kinase, or expression of p21$^{Waf/Cip-1}$, p27$^{Kip1}$, cyclin D and E2F-1. Consistent with these observations, SB218078 also inhibited ($IC_{50}$ = 450 ± 100 pM) PDGF-induced cell cycle progression.

**Conclusions and implications:** Calcitriol decreased PDGF-induced ASM cell growth by inhibiting Rb and Chk1 phosphorylation.

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**Keywords:** asthma; airway remodelling; cell cycle; hyperplasia; chronic obstructive pulmonary disease

**Abbreviations:** Ac-DEVD-pNA, Ac-Asp-Glu-Val-Asp-chromophore *p*-nitroaniline; AHR, airway hyper-responsiveness; ASM, airway smooth muscle; ATM kinase, ataxia telangiectasia mutated kinase; ATR kinase, ATM and Rad3-related kinase; BSA, bovine serum albumin; CDK, cyclin-dependent kinase; Cdc25C, cell division cycle 25 homolog C; Cdc2, cell division cycle 2 proteins; Chk, checkpoint kinase; COPD, chronic obstructive pulmonary disease; CYP24A1, cytochrome P450, family 24, subfamily A, polypeptide 1; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinases; E2F, E-2 family of DNA-binding transcription factor; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulphonyl fluoride; *p*-NPP, *p*-nitrophenyl phosphate; p27$^{Kip1}$, cyclin-dependent kinase inhibitor 1B; Rb, retinoblastoma protein; S6K1, p70/p85-S6 protein kinase 1; VDR, vitamin D receptor

## Introduction

Calcitriol (1,25-dihydroxycholecalciferol), the active metabolite of the secosteroid hormone vitamin D, plays a pivotal role in cell differentiation, immunomodulation, mineral homeostasis and cell growth (Dusso *et al.*, 2005; Brown and Slatopolsky, 2008). Through activation of the vitamin D receptor (VDR), a member of the steroid–thyroid hormone nuclear receptor subfamily, calcitriol acts as a ligand-dependent transcription factor, heterodimerizing with retinoid X receptor, that, in turn, translocates to the nucleus and binds the vitamin D response elements that modulate gene expression (Dusso *et al.*, 2005). Recent evidence also suggests that vitamin D may not only act by modulating genomic responses, but also may affect cell function non-genomically (Farach-Carson and Nemere, 2003). Although calcitriol plays a seminal role in bone and mineral metabolism, the ubiquitous expression of VDR in cells and the pleiotropic effects of calcitriol in a variety of tissues suggest an expanded role of vitamin D as a therapeutic agent in the treatment of autoimmune diseases, psoriasis and cancer (Farach-Carson and Nemere, 2003; Dusso *et al.*, 2005; Brown and Slatopolsky, 2008). Vitamin D is the focus of considerable research efforts due to cell and tissue effects.

In a variety of cell types, vitamin D manifests antiproliferative and/or pro-apoptotic actions. In some, but not all, cancer cell lines, vitamin D inhibits cell proliferation (Merke *et al.*, 1989; Iseki *et al.*, 1999; Mantell *et al.*, 2000; Bernardi *et al.*, 2002; Furigay and Swamy, 2004). In normal keratinocytes, however, calcitriol has little effect on cell growth while inhibiting keratinocytes derived from subjects who have psoriasis (Kragballe and Wildfang, 1990). In endothelial cells, calcitriol inhibits cell growth and is antiangiogenic *in vitro* and *in vivo* in a number of tumour model systems (Merke *et al.*, 1989; Iseki *et al.*, 1999; Mantell *et al.*, 2000; Bernardi *et al.*, 2002; Furigay and Swamy, 2004). In a prostate cancer model, calcitriol also has anti-tumour activity *in vivo*; however, PC3 cells are relatively insensitive to the inhibitory effects of calcitriol (Hershberger *et al.*, 2001). The mechanisms by which calcitriol inhibits growth are diverse. In most cells, the inhibition of cell growth occurs at the $G_0/G_1$ progression while in other cell types, calcitriol inhibits progression at $G_2$/M. Calcitriol inhibits growth factor-induced or constitutive activation of extracellular signal-regulated kinases (ERK)1/2 phosphorylation (Bernardi *et al.*, 2001; McGuire *et al.*, 2001). Hyperphosphorylation of the retinoblastoma protein (Rb) integrates growth signals from a variety of mitogen receptor pathways. Recent evidence also suggests that checkpoint kinase (Chk) may modulate Rb hyperphosphorylation (Verlinden *et al.*, 2007). The inhibition of cell cycle progression can also occur at the level of p21 and p27 phosphorylation, as well as in the induction of apoptosis (Deeb *et al.*, 2007; Brown and Slatopolsky, 2008). Taken together, the inhibitory effects of calcitriol on cell growth are cell and signalling pathway specific.

Asthma, a disease characterized by airway inflammation and hyper-responsiveness, may be manifested as irreversible airflow obstruction in some patients. In chronic asthma, structural alterations of the airway characterized by airway smooth muscle (ASM) hypertrophy and hyperplasia are well described (Heard and Hossain, 1973; Roth *et al.*, 2004; Woodruff *et al.*, 2004). Despite the use of glucocorticoids in the management of airway inflammation and hyper-responsiveness, there remains no specific therapy to prevent or inhibit ASM growth. Vitamin D may play a role in modulating allergen-induced airway hyper-responsiveness (AHR). Evidence suggests that vitamin D deficiency increases the susceptibility of subjects to the development of asthma, and supplementation with vitamin D decreases the severity of asthma in experimental models (Holick, 2007; 2008; Litonjua and Weiss, 2007; Taher *et al.*, 2008). The role of vitamin D in the management of asthma remains unclear. In mice deficient in VDR, allergen-induced AHR decreases in comparison to litter mate control mice, suggesting that vitamin D may promote AHR (Wittke *et al.*, 2004). In an earlier study, investigators demonstrated that serum from subjects with asthma stimulates growth of human ASM, and vitamin D decreases cell mitogenesis (Song *et al.*, 2007). Despite current evidence, the precise roles and mechanisms by which vitamin D modulates allergen-induced AHR, inflammation and ASM growth remain controversial.

In this study, we have investigated whether calcitriol inhibited human ASM cell growth derived from subjects who are normal or those with asthma. Interestingly, calcitriol, but not dexamethasone, inhibited growth factor-induced human ASM proliferation. Calcitriol also inhibited Rb protein hyperphosphorylation induced by platelet-derived growth factor (PDGF) and Chk1 phosphorylation. In an assessment of other signalling pathways mediating calcitriol effects on cell growth, activation of ERK1/2; the p70/p85-S6 protein kinase 1 (S6K1); and expression of cyclin D, p21/p27 and elongation factors 1 and 2 did not correlate with calcitriol-induced inhibition of human ASM proliferation. Calcitriol may thus offer a unique therapeutic approach in the management of diseases characterized by increased ASM mass which would include asthma and chronic obstructive pulmonary disease (COPD).

## Methods

*ASM cell culture and characterization*

Human bronchi were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. The preparation of the human ASM cells used in these studies was as previously described (Panettieri *et al.*, 1989). Briefly, a segment of the main stem bronchus at or about the carina was removed under sterile conditions and the trachealis muscle isolated. The muscle was minced, and washed in buffer containing 0.2 mM CaCl, 640 units·mL$^{-1}$ collagenase, 1 mg·mL$^{-1}$ soy bean trypsin inhibitor and 10 units·mL$^{-1}$ elastase. Enzymatic dissociation was performed for 90 min in a shaking bath at 37°C. The suspension was then filtered

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through 105 μm Nytex mesh, and the filtrate was washed with equal volumes of cold Ham's F12 media supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA). Aliquots of the cell suspension were plated at a density of $1 \times 10^4$ cells·cm$^{-2}$. The cells were cultured in Ham's F12 media supplemented with 10% serum, 100 units·mL$^{-1}$ penicillin, 0.1 mg·mL$^{-1}$ streptomycin and 2.5 μg·mL$^{-1}$ amphotericin B, and this was replaced every 72 h. Human ASM cells subcultured during the second through fifth passages were used, as these cells retain native contractile protein expression as demonstrated by immunocytochemical staining through smooth muscle specific α-actin (Panettieri *et al.*, 1989).

Smooth muscle cells obtained from subjects with asthma were obtained through the National Disease Research Interchange (NDRI) (Philadelphia, PA, USA). Briefly, cell cultures were obtained from five separate donors, and although the medical histories of the patients were limited as defined by the guidelines of NDRI, all patients had died of asthma and no patients had other systemic illnesses. To compare the growth effects in ASM cell lines derived from normal subjects and those with asthma, age and gender were matched in five cell lines derived from normal subjects who died of head trauma. The average age of the subjects who died of asthma was 38 ± 4 years, and that of normal subjects was 42 ± 8 years. Two males and three females in cell lines derived from subjects with asthma were matched with two males and three females derived from normal subjects. In all cases of comparisons between normal and asthma ASM, five distinct cell lines from each cohort were used. Overall, the subject characterization regarding age and gender showed no statistical differences between those with fatal asthma compared with normal subjects.

*Thymidine incorporation*

[$^3$H]-thymidine in human ASM cells was assessed as previously described (Panettieri *et al.*, 1989; Billington *et al.*, 2005). In brief, cells were grown in 24-well plates to near confluence and then serum deprived in 0.1% bovine serum albumin (BSA) for 24 h. Cells were pretreated with vehicle, varying doses of dexamethasone and calcitriol, and then stimulated with vehicle, PDGF or thrombin. After 16 h of stimulation, cells were labelled with 3.0 μCi [methyl-$^3$H]-thymidine (1 μCi·mL$^{-1}$) and incubated for an additional 24 h. Cells were then washed with phosphate-buffered saline (PBS), lysed with 20% trichloracetic acid, aspirated on to filter paper and counted in scintillation vials. In separate studies, cell proliferation assays were performed as described previously (Krymskaya *et al.*, 2000). ASM cells were grown in six-well plates (triplicate wells per condition) to near confluence, and then serum starved and stimulated as described earlier for analyses of thymidine incorporation. After 48 h of stimulation, the cells were harvested, and viable cells were counted with an automated cell counter (BiCell, Beckman Coulter, Fulton, CA, USA).

*Cell cycle analysis*

The ASM cells were seeded and grown to confluence in 10% FBS and F-12 media as described earlier. The cells were starved for 48 h in serum-free media after which any experimental drug treatments were performed. The plates were aspirated, washed with PBS and replaced with 2 mL per well trypsin (0.05%), and incubated at 37°C for 5 min. The detached cells were transferred to a microcentrifuge tube and pelleted. The cells were then resuspended in 1 mL CycleTest Buffer Solution (CycleTest Plus Kit, BD Biosciences, San Jose, CA, USA) and either frozen at –80°C for future analysis or resuspended in buffer at room temperature. After incubating for 10 min, buffer was added and mixed as per manufacturer's instructions.

*Analysis of propidium iodide (PI)-stained nuclei*

On a BD FACS Canto flow cytometer, debris and large clumps were gated by scatter (Billington *et al.*, 2005). Nuclei aggregates in PI emission range were performed by FL2-A/FL2-W crossplot analysis using Cell Quest Pro Software. The DNA contents of the nuclei were compared on a linear FL2-A histogram. Data were analysed with Verity ModFit LT 3.0 Software for cell cycle phase percentages.

*Caspase 3 analysis and apoptosis*

The effects of calcitriol on ASM cell apoptosis were evaluated by a Clontech ApoAlert Caspase-3 Colorimetric Assay Kit (Clontech Labs, Palo Alto, CA, USA). Growth-arrested ASM cells treated with 10 μM of calcitriol, 10 ng·mL$^{-1}$ of PDGF or a combination were lysed in cold lysis buffer provided with the kit. For control experiments, caspase-3 was induced by treating cells with 40 μM ceramide (Sigma-Aldrich, St Louis, MO, USA) (Krymskaya *et al.*, 2000). Lysates were incubated at 37°C for 1 h in a reaction buffer containing 50 μM of Ac-Asp-Glu-Val-Asp-chromophore *p*-nitroaniline (Ac-DEVD-pNA) and analysed on an enzyme-linked immunosorbent assay (ELISA) plate reader at 405 nm. All data obtained were normalized against the total protein concentration determined by a Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

*Reverse transcription (RT)–polymerase chain reaction (PCR) analysis*

Total RNA was extracted from human ASM cells using RNEasy Mini Kit (Qiagen, Valencia, CA, USA) as previously described (Tliba *et al.*, 2006). In preliminary experiments, we determined for each primer pair the melting temperature and number of amplification cycles necessary to yield the appropriate hybridization signal. PCR of cytochrome P450, family 24, subfamily A, polypeptide 1 enzyme (CYP24A1), VDR and glyceraldehyde-3-5 phosphate dehydrogenase (GAPDH) was performed using our previously published primers (Banerjee *et al.*, 2008).

*Immunoblot analysis*

Immunoblot analysis of dose-dependent effects of calcitriol and dexamethasone on phosphor-thr202/tyr 204 p42/p44, S6K1, phospho-Rb protein, cyclin D, p21$^{Waf/Cip-1}$, p27$^{Kip1}$, E2F-1 to E2F-4 and β-actin was performed as described (Billington *et al.*, 2005; Guo *et al.*, 2005). Serum-starved ASM cells were

washed with PBS and resuspended in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 1 mM $Na_3VO_4$, 10 μg·mL$^{-1}$ aprotinin and leupeptin. Proteins were analysed on a 12.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and blotted onto a nitrocellulose membrane. The membranes were blocked in 3% BSA in Tris-buffered saline, and incubated with primary antibodies as recommended by the manufacturer. Following incubation with the appropriate peroxidase-conjugated secondary antibody (Roche Molecular Biochemicals, Minneapolis, MN, USA), the bands were visualized by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and autoradiographed. To ensure equal loading, the membranes were stripped and reprobed with β-actin antibodies or E2F-1 antibodies to confirm equal protein loadings.

*S6K1 activity assay*

*In vitro* S6K1 activity assay was performed as described previously (Goncharova *et al.*, 2002). Briefly, serum-free, medium-maintained cells were washed twice in ice-cold PBS, and then lysed in lysis buffer [20 mM Tris, pH 8.0; 150 mM NaCl; 5 mM EGTA; 1 mM EDTA; 10 mM $Na_4P_2O_7 \times 10H_2O$; 1 mM benzamidine; 1% NP-40; 10 mM *p*-nitrophenyl phosphate (*p*-NPP); 0.1 mM PMSF]. After incubation for 30 min at 4°C, lysates were centrifuged at 18 500× *g* for 10 min. Supernatants were incubated with 2 μg of anti-S6K1 antibody with gentle rocking overnight at 4°C. The immunocomplexes were collected by 50 μL of protein A-Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) for 2 h at 4°C. The immunoprecipitates were washed twice with ice-cold cell lysis buffer, twice with lysis buffer without NP-40 and twice with an assay dilution buffer [20 mM MOPS, pH 7.2; 25 mM β-glycerol phosphate; 5 mM EGTA; and 1 mM dithiothreitol (Sigma Chemical Co.)]. The immunoprecipitates/protein A-Sepharose beads were then resuspended in assay dilution buffer containing 50 μM substrate peptide, 4 μM protein kinase C inhibitor peptide, 0.4 μM protein kinase A inhibitor peptide and [γ-$^{32}$P] ATP (NEN DuPont, Boston, MA, USA). The samples were incubated for 10 min at 30°C, and then 20 μL of the reaction mixture was spotted onto p81 phosphocellulose filters, which were washed three times with 0.75% phosphoric acid and once with acetone. The radioactivity of samples was measured using a Beckman LS 6500 scintillation counter.

*Statistical analysis*

Data are presented as means ± standard error of the mean (SEM). In the instances where cells derived from subjects with fatal asthma are compared with those derived from normal subjects, four cell lines in each group matched by age and gender were used. Individual data points from a single experiment were calculated as the mean value from three or six replicate observations from tritiated thymidine incorporation or from three replicates for apoptosis assays and cell cycle determinations. For immunoblot analyses, band intensities representing signals from secondary antibody were quantitated using Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA). These values were normalized to values determined from β-actin and compared among stimuli in experimental groups. Statistically significant differences among groups were assessed by either analysis of variance (ANOVA) with Fisher's protected least significant differences *post hoc* analysis (Stat; Abacus Concepts, Berkeley, CA, USA) or by *t*-test on prepared samples, with $P$ values $\leq 0.05$ sufficient to reject the null hypothesis. With multiple comparisons, ANOVA with a Bonferroni–Dunn correction was also used. All immunoblot analyses were done over a range of loading such that comparisons on the linear portion of loading to response were provided.

*Materials*

Tissue culture media, reagents and primers for PCR were obtained from Invitrogen (Carlsbad, CA, USA). PDGF and thrombin were sourced from Calbiochem (San Diego, CA, USA). Calcitriol (1α, 25-dihydroxy vitamin $D_3$) and dexamethasone were obtained from Cayman Chemical (Ann Arbor, MI, USA) and Sigma respectively. Antibodies for E2F-1, p18, phospho-Rb, cyclin-A, phospho-Chk1, phospho-Cdc25C and phospho-Cdc2 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies for E2F-2, E2F-3, E2F-4, p19, p27, p107 and p130 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-S6K1 antibody was purchased from Santa Cruz Biotechnology. S6K1 assay kit was purchased from Upstate Cell Signaling (Lake Placid, NY, USA).

All nomenclature for the therapeutic agents, drugs, proteins and receptors used in the study follows the guidelines in Alexander *et al.* (2008).

## Results

*ASM DNA synthesis induced by growth factors and VDR activation by calcitriol*

To characterize the effects of calcitriol on ASM cell proliferation, cultured ASM cells derived from normal subjects and those with asthma were serum deprived and then stimulated with PDGF, thrombin or a combination. As shown in Figure 1A (upper panel), both PDGF and thrombin induced DNA synthesis in ASM cells, and the combination produced additive effects. In cells derived from subjects with asthma, PDGF, thrombin and the combination enhanced DNA synthesis as compared with DNA synthesis in ASM derived from subjects without asthma. The time course and concentrations for PDGF (10 ng·mL$^{-1}$) and thrombin (1 U·mL$^{-1}$) stimulation of DNA synthesis are those found in bronchoalveolar lavage fluid in subjects with asthma and are those described in our previous studies (Panettieri *et al.*, 1995). In parallel experiments, PDGF, thrombin and the combination increased ASM cell number by 32 ± 3%, 15.2 ± 3% and 42 ± 4%, respectively, as compared to diluent-treated normal ASM cells. In asthma ASM, PDGF, thrombin and the combination augmented cell proliferation by 10 ± 2%, 8.1 ± 9% and 21 ± 4% as compared to normal ASM. All conditions were statistically different ($P < 0.05$) comparing normal and asthma ASM cell numbers stimulated by PDGF, thrombin and the combination.

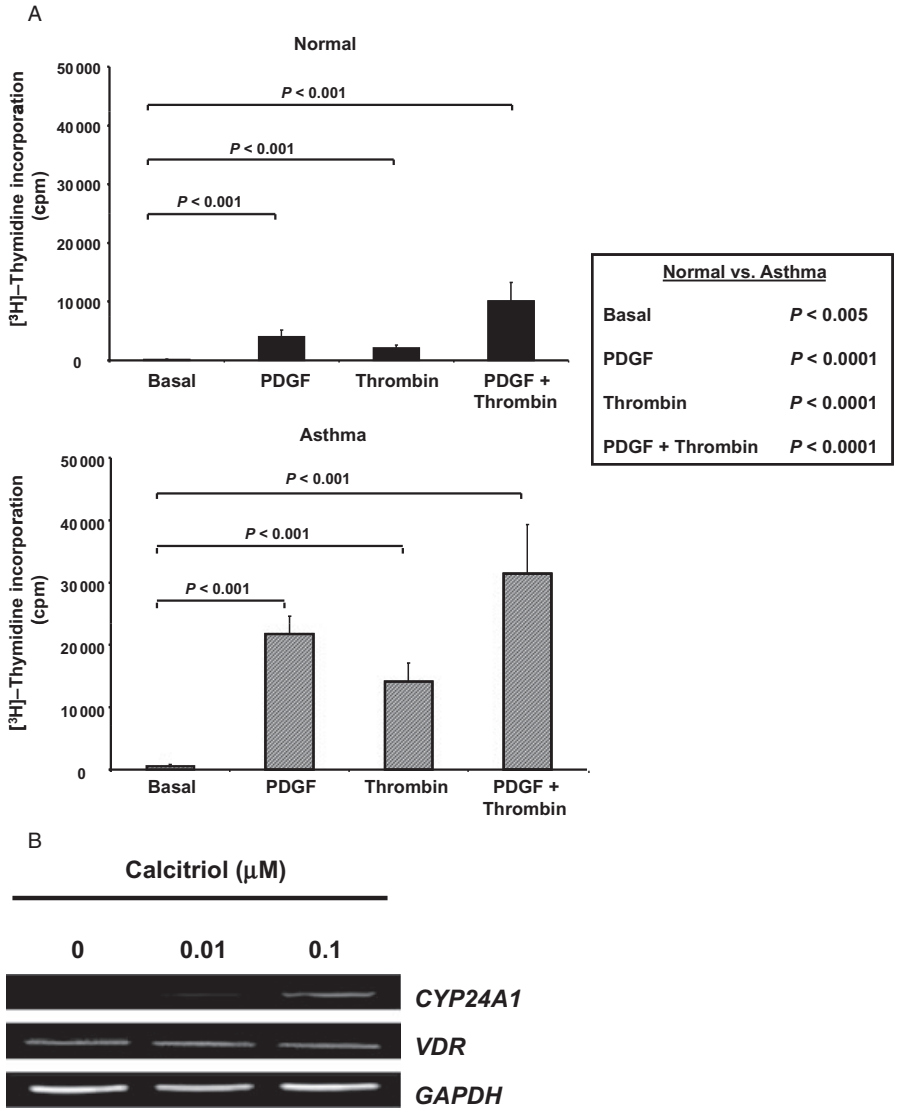


**Figure 1** Airway smooth muscle (ASM) DNA synthesis and vitamin D receptor (VDR) activation. (A) [$^3$H]-thymidine incorporation was measured in quiescent ASM cells derived from normal subjects and those with asthma. Platelet-derived growth factor (PDGF), thrombin and the combination markedly enhanced [$^3$H]-thymidine incorporation in both normal and asthma ASM. There was a dramatic enhancement in thymidine incorporation in asthma ASM as compared with normal ASM stimulated by PDGF or thrombin. The data represent mean ± standard error of the mean from four separate cell lines with each condition representing a minimum of six replicates. Statistics were performed using analysis of variance with Bonferroni–Dunn correction; $P < 0.05$ considered significant. (B) Quiescent ASM cells from normal subjects were stimulated with varying concentrations of calcitriol, and expression of CYP24A1, VDR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) total mRNA was determined by reverse transcription–polymerase chain reaction. Data shown are representative of three separate experiments from four different cell lines derived from normal subjects or those with asthma.

To address whether ASM cells expressed VDR and whether activation of the receptor with calcitriol altered gene expression, RT–PCR was performed in ASM treated with varying concentrations of calcitriol as shown in Figure 1B. Calcitriol induced expression of CYP24A1 (cytochrome P450, family 24, subfamily A, polypeptide 1), a hydroxylase that metabolizes calcitriol. CYP24A1 expression requires calcitriol and serves as a biomarker of VDR activation (Bernardi *et al.*, 2002). Compared to total mRNA of VDR or GAPDH, increasing doses of calcitriol had little effect on VDR or GAPDH expression. The expression of CYP24A1, VDR and GAPDH in response to varying doses of calcitriol was also examined in normal and asthma ASM; there was no difference in expression levels between these cell lines (data not shown). Collectively, these data suggest that calcitriol specifically enhances expression of known VDR-regulated genes and that calcitriol itself had little effect on altering mRNA expression of its cognate receptor.

### *Calcitriol, but not dexamethasone, inhibits mitogen-induced DNA synthesis*

To characterize whether calcitriol modulated growth factor-induced DNA synthesis, cells were serum-starved and then stimulated with increasing doses of calcitriol in the presence of either PDGF or thrombin. Effects of calcitriol on DNA synthesis in normal and asthma ASM cells were also com-

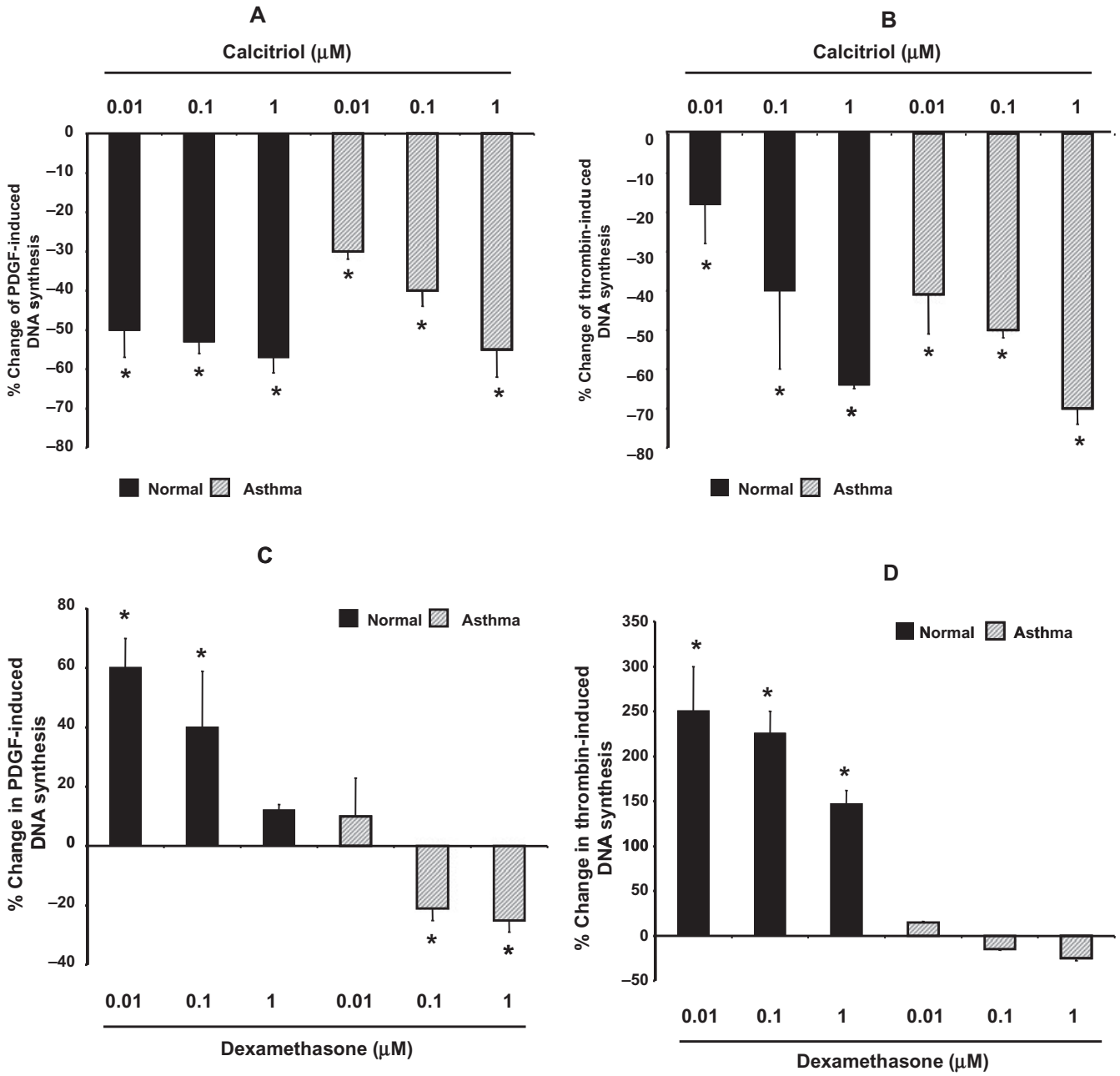


**Figure 2** Calcitriol, but not dexamethasone, inhibits DNA synthesis in airway smooth muscle (ASM) cells. ASM were grown to near confluence, serum-deprived and then stimulated with growth factors. Thymidine incorporation was determined as described in Methods. Calcitriol inhibited platelet-derived growth factor (PDGF)-induced (A) or thrombin-induced (B) DNA synthesis in ASM from normals and subjects with asthma. (C) Dexamethasone augmented PDGF-induced DNA synthesis in normal ASM, while modestly inhibiting DNA synthesis in asthma cells. (D) Dexamethasone augmented thrombin-induced DNA synthesis in ASM from normal subjects, but had no effect on asthma ASM. All data were normalized to maximum stimulation by a growth factor after the basal thymidine incorporation counts were subtracted. All data in (A) and (B) represent $P$ values > 0.05 when compared to maximum PDGF-induced DNA synthesis (100%). In (C) and (D), growth augmentation by dexamethasone on PDGF- and thrombin-induced DNA synthesis was significant at concentrations of 0.1 and 1 μM for PDGF, and at all tested concentrations for thrombin-induced DNA synthesis ($P < 0.05$). Dexamethasone effects in asthma ASM cells were not different in comparison to thrombin-induced DNA synthesis, and significant at concentrations of 1 and 10 μM dexamethasone plus PDGF when compared with responses stimulated by PDGF alone ($P > 0.05$). All data represent means ± SEM. Significance was assessed using analysis of variance with Bonferroni–Dunn correction, with significance demonstrated at $P < 0.05$.

pared with those observed in cells treated with dexamethasone. The effects of calcitriol on ASM growth were compared to dexamethasone because glucocorticoids are a mainstay in the management of asthma, and glucocorticoids inhibit growth of some cell types (Panettieri, 2008). As shown in Figure 2A, calcitriol maximally inhibited PDGF-induced DNA synthesis by about 60% in either normal or asthma ASM. Thrombin-induced DNA synthesis in normal or asthma ASM

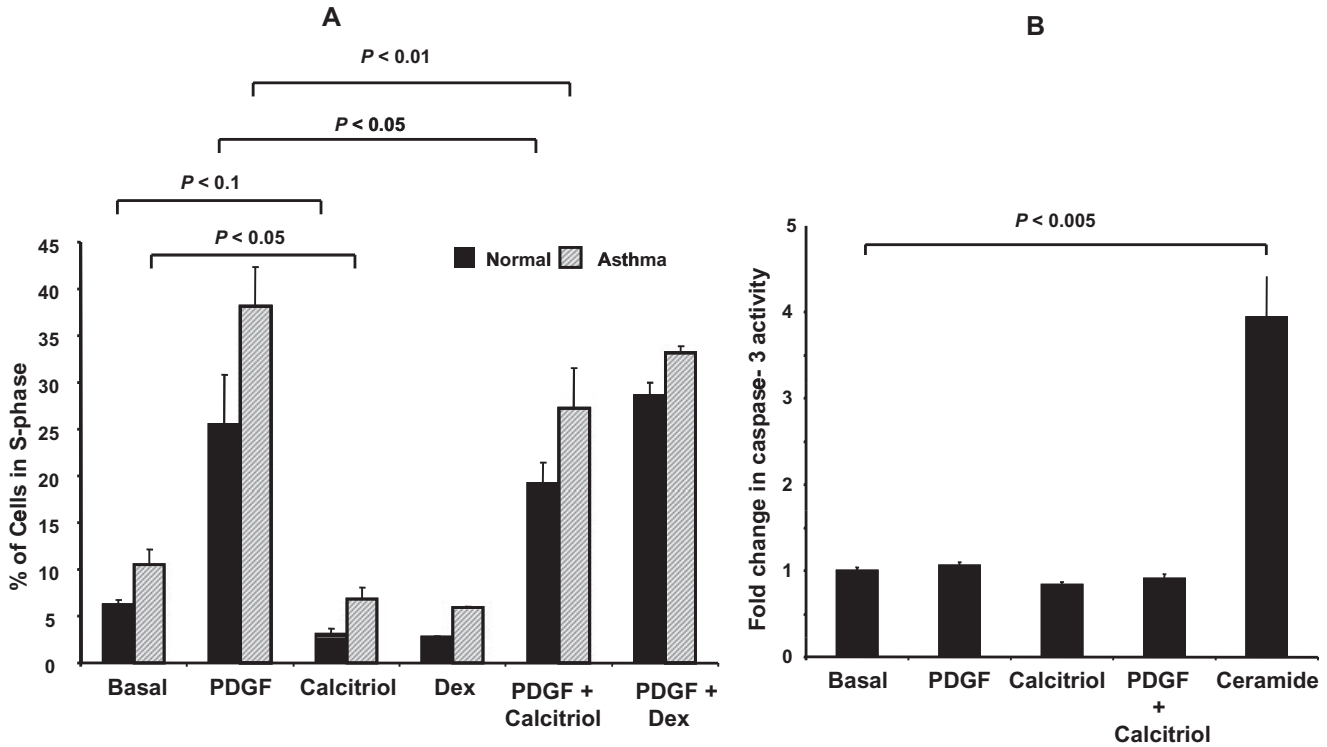


**Figure 3** Calcitriol inhibited cell cycle progression but had no effect on airway smooth muscle (ASM) apoptosis. (A) Quiescent ASM cells were stimulated with platelet-derived growth factor (PDGF), calcitriol, dexamethasone or the combination, and after 24 h, flow cytometry and characterization of cells entering the S phase of the cell cycle were performed as described in Methods. (B) Calcitriol had no effect on ASM apoptosis. ASM cells were stimulated with PDGF, calcitriol, the combination or ceramide for 48 h, and caspase-3 activity was measured as described in Methods. Ceramide served as a positive control demonstrating induction of apoptosis as measured by caspase-3 activity in ASM cells. All data represent means ± SEM. Each data point represents a minimum of four replicates from four separate cell lines derived from normal subjects and those with asthma.

cells was also inhibited ($P < 0.05$) by calcitriol, as shown in Figure 2B. The concentration to inhibit 50% ($IC_{50}$) of thrombin- or of PDGF-induced DNA synthesis by calcitriol was 520 ± 52 nM. In contrast to the effect of calcitriol on DNA synthesis, dexamethasone (Figure 2C and D) promoted PDGF-induced DNA synthesis in normal ASM, but had a modest effect at high doses in inhibiting DNA synthesis in asthma ASM cells. Dexamethasone also augmented thrombin-mediated DNA synthesis in a concentration-dependent manner while having little effect on ASM derived from subjects with asthma. Collectively, these data showed that calcitriol inhibited growth factor-induced DNA synthesis in ASM from normals and subjects with asthma. In contrast to dexamethasone, calcitriol was markedly more effective in inhibiting DNA synthesis.

*Calcitriol inhibits growth factor-induced cell cycle progression, but has little effect on ASM apoptosis*

To investigate whether PDGF-induced cell cycle progression was modulated by calcitriol or by dexamethasone, quiescent ASM cells were treated with PDGF in the presence and absence of 1 μM calcitriol or 10 μM dexamethasone. Cell cycle analysis was then characterized by double staining with DAPI and PI, and flow cytometry was performed as described in the Methods. As shown in Figure 3A, PDGF promoted cell cycle progression of ASM derived from normal subjects and those with asthma. Consistent with the [$^3$H]-thymidine incorporation and cell counting experiments, asthma cells entered the S phase of the cell cycle more readily than those derived from normal subjects. Even in the basal, serum-deprived state, asthma ASM cells were about twice as likely to enter the S phase than those derived from normal subjects (Figure 3A, $P < 0.05$). Calcitriol inhibited PDGF-induced cell cycle progression, equally, in ASM cells from normal (26 ± 0.8%, $P < 0.05$) or asthma subjects (28 ± 0.2%, $P < 0.05$), while dexamethasone had no effect. Taken together, these data suggest that calcitriol, but not dexamethasone, inhibited PDGF-induced cell cycle progression, as determined by the numbers of cells entering the S phase of the cell cycle.

As ASM growth may be an interplay between pro-mitogenic and apoptotic signals, we next addressed whether calcitriol-induced apoptosis was manifested as inhibition of mitogenesis. As shown in Figure 3B, caspase-3 activity was measured in cells stimulated with PDGF, calcitriol or the combination. In previous studies, we have demonstrated that 1 μM ceramide induces ASM cell apoptosis (Ammit *et al*., 2001). Calcitriol had little effect on caspase-3 activity alone or in combination with PDGF, whereas ceramide markedly enhanced caspase-3 activity. In parallel, calcitriol had little effect on apoptosis using two-wavelength flow cytometric analysis (data not shown). These data suggest that calcitriol inhibited ASM cell proliferation by preventing cell cycle progression most likely

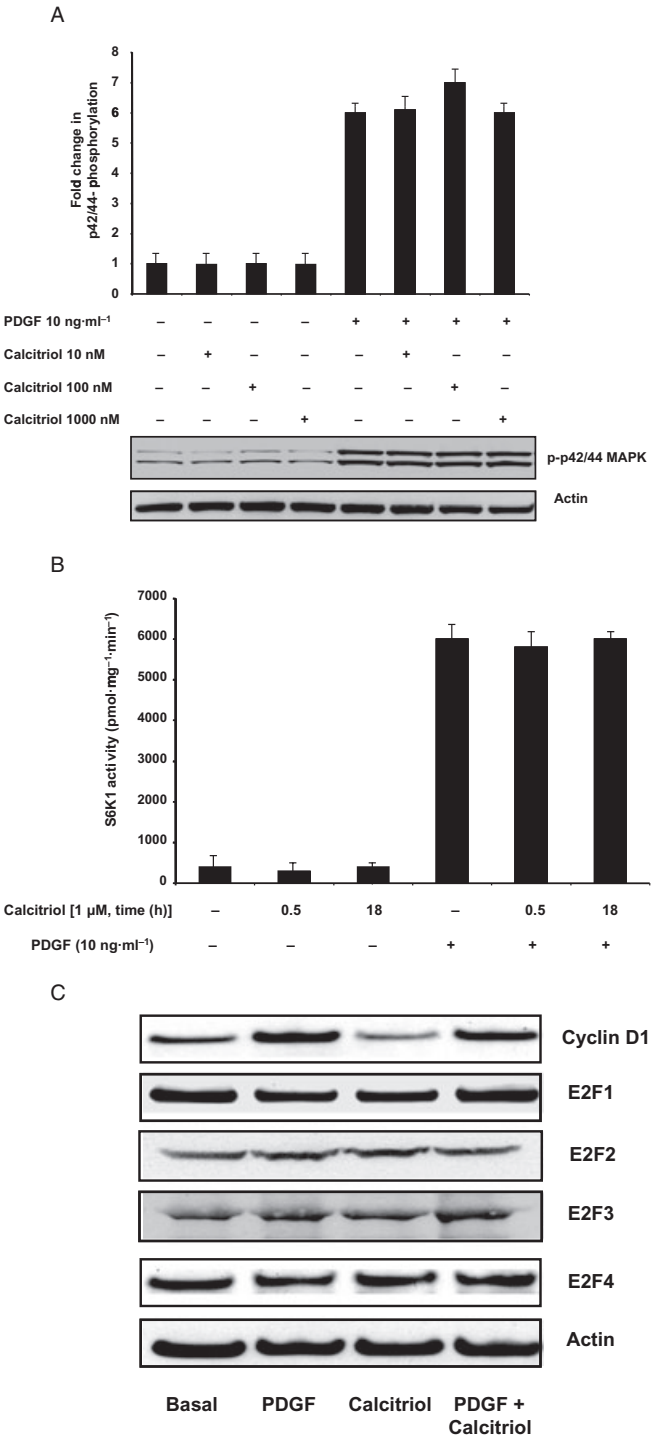


**Figure 4** (A) Platelet-derived growth factor (PDGF) induced p42/44 mitogen-activated protein kinase (MAPK) activation, but calcitriol had no little effect. Quiescent airway smooth muscle (ASM) cells from normal subjects were pretreated for 30 min with calcitriol at varying concentrations and then with PDGF (10 ng·mL$^{-1}$) for 10 min. Activation of p42/44 MAPK was characterized by immunoblot analysis as described in Methods. The bar graphs represent quantitation of the displayed immunoblots. Parallel experiments performed in ASM derived from subjects with asthma were identical to these results. Equal protein loadings were confirmed in immunoblots stripped of primary antibodies and re-probed using a β-actin antibody (data not shown). These data represent means ± standard deviations from four separate cell lines derived from normal subjects. (B) Calcitriol had no effect on S6K1 activation by PDGF. Ribosomal S6K1 activation was determined in quiescent ASM cells pretreated with calcitriol (1 μM) for 30 min or 18 h, and then stimulated with PDGF (10 ng·mL$^{-1}$). PDGF induced S6K1 activation; however, calcitriol had no effect. There was no difference in S6K1 activation induced by PDGF in ASM derived from normals and from subjects with asthma. These data are representative of three separate experiments from four different ASM cell lines. (C) Calcitriol had no effect on cyclin D1 or E2F protein expression in ASM. ASM monolayers from normal subjects were treated with 10 ng·mL$^{-1}$ PDGF, 1 μM calcitriol or the combination for 18 h. Cells were then lysed, and immunoblot analysis was performed as described in Methods. Antibodies specific for cyclin D1, E2F1–E2F4 and β-actin were used to characterize immunoblot analysis. All blots were stripped and then probed with antibodies to β-actin that serve as a control for total protein loading per well. There was no difference in protein expression profile among normal ASM and those derived from subjects with asthma. The data are representative of a single experiment repeated in triplicate in four separate cell lines.

at the $G_0/G_1$ checkpoint, thus preventing cells from entering the S phase of the cell cycle.

*PDGF induces phosphorylation of p42/44 mitogen-activated protein kinase and ribosomal S6K1*

To dissect whether p42/44 mitogen-activated protein kinase (p42/44 MAPK) and S6K1 activation induced by PDGF was modulated by calcitriol, quiescent ASM cells were stimulated with PDGF in the presence and absence of varying doses of calcitriol. In previous studies, we have demonstrated that p42/44 MAPK and ribosomal S6K1 activation are required for ASM cell proliferation (Krymskaya *et al*., 1999). As calcitriol inhibited PDGF-induced ASM proliferation, we reasoned that calcitriol may also have inhibited activation of these mitogenic signalling pathways. As shown in Figure 4A, PDGF stimulation markedly enhanced phosphorylation of p42/44 MAPK; however, pretreatment of cells with varying concentrations of calcitriol had little effect on phosphorylation of MAPK by PDGF stimulation. In separate experiments, the effect of calcitriol on PDGF-induced S6K1 activation was determined. As shown in Figure 4B, PDGF markedly enhanced S6K1 activation in quiescent ASM cells. Pretreatment of ASM with calcitriol for 30 min or 18 h had little effect on PDGF-induced S6K1 activation. These data suggest that although calcitriol inhibited ASM cell proliferation induced by PDGF, calcitriol had no effect on PDGF-induced p42/44 MAPK or S6K1 activation.

*Calcitriol has no effect on PDGF-induced cyclin D1 or E2F2 expression, but prevents PDGF-induced hyperphosphorylation of Rb protein*

In ASM cells, cyclin D1 and the E2F family of transcription factors modulate growth factor-induced mitogenesis (Bauerfeld *et al*., 2001; Tliba *et al*., 2003). The members of the E2F family of transcription factors play a critical role in the control of cell cycle progression. In addition, this family has cyclin-binding domains that preferentially interact with Rb protein in a cell cycle-dependent manner. As calcitriol inhibited ASM cell growth, we investigated whether calcitriol modulated PDGF-induced expression of these critical mitogenic signalling molecules. As shown in Figure 4C, PDGF induced cyclin D1 expression; however, calcitriol had no effect on basal cyclin D1 expression or that induced by PDGF.

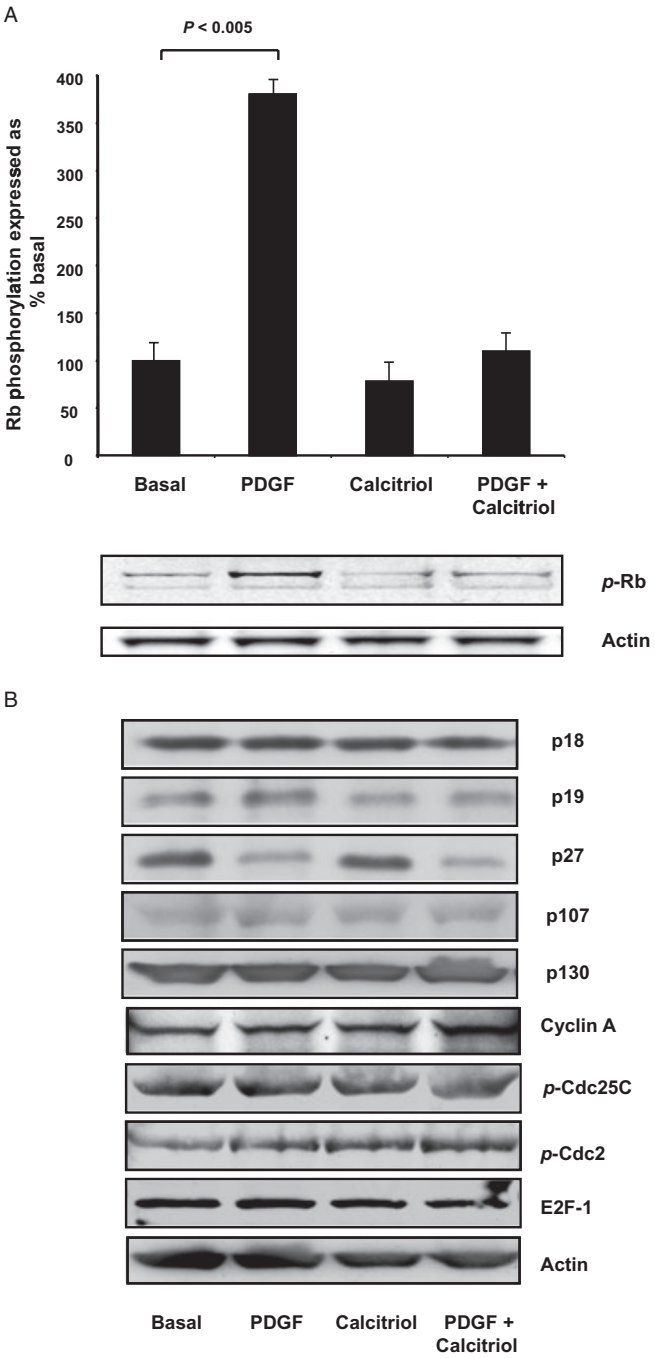


**Figure 5** (A) Calcitriol inhibited platelet-derived growth factor (PDGF)-induced hyperphosphorylation of retinoblastoma (Rb) protein. Quiescent airway smooth muscle (ASM) cells derived from normal subjects were stimulated for 12 h with PDGF (10 ng·mL$^{-1}$) in the presence and absence of a 30 min pretreatment with calcitriol (1 μM). Cells were lysed, and the supernatant was analysed by gel electrophoresis and immunoblot analysis using specific antibodies to phospho-Rb protein or actin. The immunoblot analysis is representative of triplicate experiments performed in four different cell lines derived from normal and subjects with asthma. The data are plotted as Rb phosphorylation as % control. Statistical analysis was performed examining the PDGF effects versus basal in multiple gels in multiple cell lineages. (B) Lack of effects of calcitriol on inhibitors of cell cycle progression. Confluent ASM cells were stimulated with PDGF (10 ng·mL$^{-1}$), calcitriol (1 μM) or the combination. Cells were then lysed and the protein extracted. Immunoblot analysis was performed using specific antibodies to the cell signalling molecules as described in the Methods section. In each case, the immunoblots were stripped and reprobed with a β-actin antibody to demonstrate equal loading. Calcitriol had no effect on PDGF-induced effects on inhibitors of cell cycle progression.

In parallel experiments, expression of the E2F family of transcription factors was examined in the presence and absence of PDGF and calcitriol. Calcitriol had no effect on E2F2 expression in the presence and absence of PDGF. There was also no difference in PDGF-induced cyclin D1 expression in ASM derived from normal subjects and those with asthma (data not shown). These data suggest that the mechanism by which calcitriol inhibited ASM cell proliferation did not involve cyclin D1 or the E2F family of transcription factors.

We and others have demonstrated that hyperphosphorylation of Rb protein is necessary for mitogen-induced cell cycle progression in ASM cells (Tliba *et al.*, 2003). To address whether calcitriol inhibited hyperphosphorylation of Rb protein, ASM cells were made quiescent and then stimulated with PDGF in the presence and absence of 1 μM calcitriol. As shown in Figure 5A, PDGF markedly enhanced hyperphosphorylation of Rb protein, while calcitriol prevented PDGF-induced Rb phosphorylation.

*Calcitriol has no effect on cell cycle inhibitor proteins, but specifically inhibits phosphorylation of Chk1*

Calcitriol inhibited ASM cell proliferation, at least in part, by decreasing PDGF-induced hyperphosphorylation of Rb protein. As phosphorylation of Rb protein can be modulated by cell cycle inhibitors, mainly Cip/Kip, an INK-4 family of inactive cyclin-dependent kinases (CDKs), we examined whether members of the INK-4 family (p18, p19) as well as the Cip/Kip family members (p27) mediated calcitriol's effects on hyperphosphorylation of Rb protein and cell growth. As shown in Figure 5B, p27, but not p18, p19, p107 or p130, expression is elevated in quiescent ASM cells, and upon stimulation with PDGF, p27 expression surprisingly decreases. Calcitriol had no effect on the inhibition of p27 expression or the INK-4 family. In parallel experiments, we addressed whether modulators of an intra S phase checkpoint would mediate calcitriol's effects on ASM growth. As shown in Figure 5B, cyclin A expression as well as phosphorylation of Cdc25C and Cdc2 were unaffected by either PDGF or calcitriol.

To address whether other protein kinases may mediate the growth inhibitory effects of calcitriol, we examined whether Chk1 phosphorylation was altered by PDGF and calcitriol. Chk1 and Chk2 primarily act as inhibitors of cell cycle progression and promote DNA repair after radiation injury (Enders, 2008). New evidence suggests that Chk1 and Chk2 may also play a role in promoting cell cycle progression in non-malignant cells (Zachos and Gillespie, 2007; Enders, 2008). As demonstrated in Figure 6A, pretreatment of ASM cells with the Chk1 specific inhibitor (SB218078; 0.1–100 nM) inhibited PDGF-induced S-phase progression concentration dependently. Further, PDGF enhanced Chk1 phosphorylation

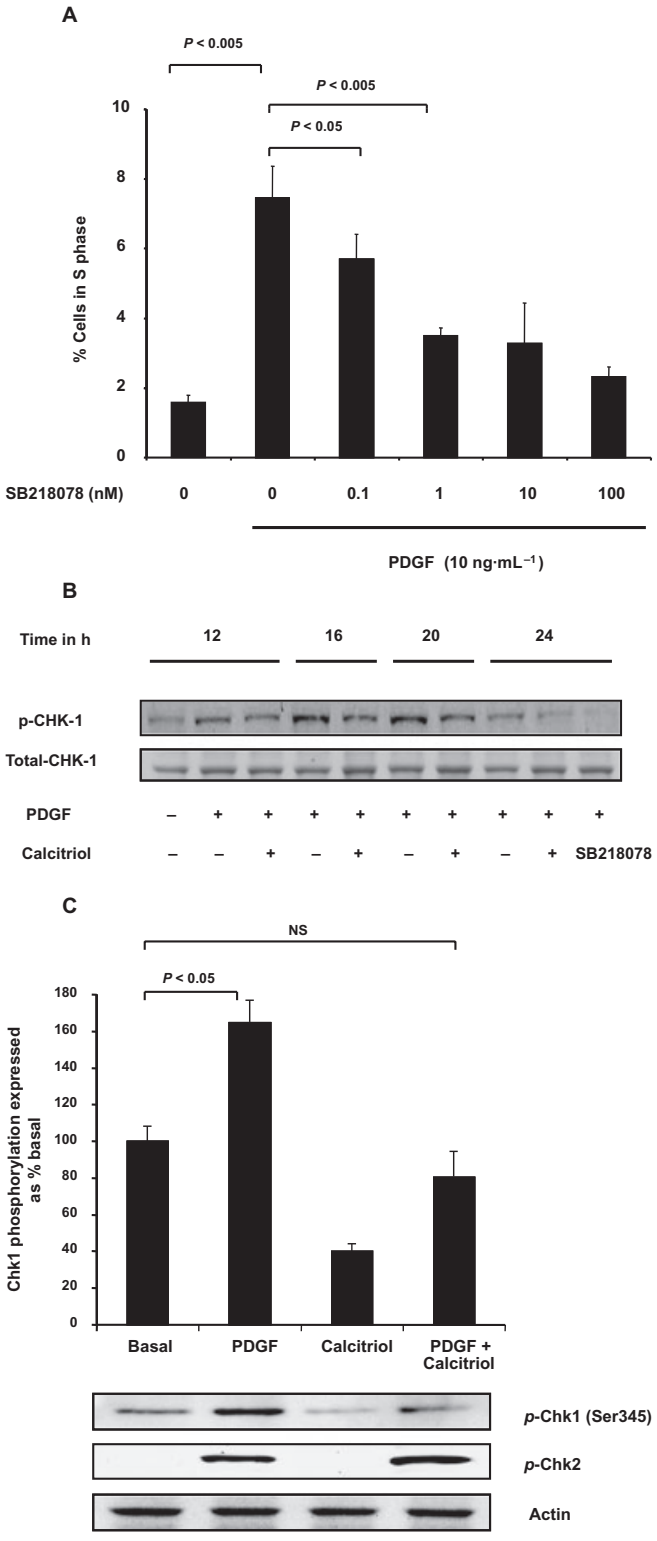


**Figure 6** (A) Chk1 activity was essential for platelet-derived growth factor (PDGF)-induced cell cycle progression. Quiescent airway smooth muscle (ASM) cells from normal subjects were treated with PDGF (10 ng·mL$^{-1}$) for 24 h following pretreatment with varying concentrations (0.1–100 nM) of Chk1 inhibitor (SB218078). Following the 24 h time period, the S phase cell cycle progression was analysed by flow cytometry as described in the Methods section. The data are representative of a single experiment repeated in triplicate in four separate cell lines derived from normal and subjects with asthma. (B) Calcitriol inhibited Chk1 phosphorylation in PDGF-stimulated ASM cells. Quiescent ASM cells were pretreated with calcitriol (1 μM) or SB218078 for 2 h before stimulating with PDGF (10 ng·mL$^{-1}$) for the indicated times. Immunoblot analysis used specific antibodies to phospho-Chk1. Immunoblots were stripped and re-probed with a β-actin antibody to demonstrate equal loading. PDGF-induced Chk1 phosphorylation was inhibited by calcitriol at 1 μM in a time-dependent manner. (C) Densitometric analysis of Chk1 and Chk2 phosphorylation after 24 h of PDGF treatment in calcitriol-treated and untreated ASM cells. Data are expressed as % basal phospho-Chk1 after normalizing for actin. Pretreatment of ASM cells with calcitriol (1 μM) markedly inhibited PDGF-induced Chk1 phosphorylation with no effect on Chk2 phosphorylation. The data are representative of repetitive densitometric measurements of triplicate immunoblots from four separate ASM cell lines derived from normal and subjects with asthma.

in a time-dependent manner, and pretreatment of ASM with calcitriol at 1 μM markedly inhibited PDGF-mediated Chk1 phosphorylation as shown in Figure 6B. The phosphorylation of Chk1 was specific because Chk2 phosphorylation was unaffected (Figure 6C). Collectively, these data suggest that calcitriol may inhibit PDGF-induced ASM proliferation at the level of hyperphosphorylation of Rb protein, as well as by diminishing the phosphorylation of Chk1.

## Discussion

Calcitriol modulates pleiotropic cellular effects in a tissue-specific manner. The effects of calcitriol on bone and calcium metabolism have long been known; however, recent interests have focused on calcitriol as a modulator of inflammation and cell proliferation. In the current studies, calcitriol inhibited ASM growth in ASM derived from normal subjects and from those with asthma. Despite the widespread use of glucocorticoids to treat asthma and airway inflammation, dexamethasone had little effect on inhibition of ASM cell proliferation in comparison to the effects of calcitriol.

Calcitriol inhibits proliferation of some, but not all, transformed cells, and the precise signalling pathways mediating calcitriol's effects on growth are cell and tissue specific. In some cancers, calcitriol inhibits p42/44 MAPK activation and can inhibit traversal of the cell cycle at the $G_0/G_1$ checkpoint. In other cell types, calcitriol induces apoptosis or may inhibit traversal of S phase or $G_2/M$ progression. In ASM cells, calcitriol had little effect on p42/44 MAPK and ribosomal S6K1 activation, but markedly inhibited hyperphosphorylation of Rb. Because the anti-proliferative effects of calcitriol in ASM could be mediated by preventing phosphorylation of Rb, we next examined known modulators of Rb phosphorylation such as cyclin D. Surprisingly, calcitriol had no effect on PDGF-induced cyclin D expression. In other cell types, growth factor-induced cyclin D expression was markedly inhibited by calcitriol (Iseki *et al.*, 1999). As calcitriol inhibited ASM cell proliferation and cell cycle progression that appeared to occur prior to the S phase, we determined whether specific cell cycle inhibitors were affected by calcitriol. The INK-4 or Cip/Kip cell cycle inhibitors play a critical role in mediating effects of cyclin D on Rb hyperphosphorylation. In ASM cells, however,

calcitriol had no effect on the inhibition of the INK-4 cell cycle inhibitors. To confirm that S phase progression checkpoints were unaffected, we then examined the expression of cyclin A as well as other downstream Rb phosphorylation products of the E2F family. Calcitriol had no effect on cyclin A or E2F expression, suggesting alternative sites by which calcitriol mediates its anti-proliferative effects in ASM cells.

The orderly activation of signalling events promotes cell cycle progression through specific checkpoints that can also serve to inhibit cell growth. A critical checkpoint that prevents cell cycle progression after genotoxic stress such as radiation or mitotic spindle toxin exposure involves the activation of a family of serine–threonine kinases known as checkpoint kinase 1 and 2 (Chk1, Chk2). Chk1 inhibits CDK activity through destabilization of the CDK-activated Cdc25A. DNA double-strand breaks that impede progression of replication forks are detected by members of the PI3-kinase family of enzymes, ATM and ATR. Activation of ATM and ATR then activates 'effector' kinases Chk1 and Chk2. Chk1 is primarily activated by DNA replication defects through generation of single-stranded DNA, while Chk2 in concert with ATM is activated by double-stranded DNA breaks. Thus, in interrupted cell cycle progression due to DNA damage, Chk1 and Chk2 delay DNA replication, stabilize DNA replication forks and prevent cell death by blocking mitotic entry. New evidence, however, suggests that activation of Chk1 early in the cell cycle may play a pivotal role in promoting cell cycle progression and growth. In some cells, Chk1 activation maintains basal transcription of cell cycle genes, thus serving as a positive factor in cell cycle progression (Maude and Enders, 2005). Further, Chk1 can phosphorylate Rb that enhances E2F binding. This function is consistent with evidence that Chk1 is preferentially expressed in cycling cells, may be activated by E2F proteins (Ren *et al.*, 2002) and is induced by CDK2 activity (Maude and Enders, 2005).

We found that PDGF induced phosphorylation of Chk1, a protein kinase downstream from ATM/ATR. The activation of Chk1 and Chk2 involves phosphorylation at specific serines by ATR, and Chk1 is also a substrate for Akt. In the current study, PDGF promoted Chk1 phosphorylation, and specific inhibition of Chk1 markedly curtailed PDGF-induced S-phase cell cycle progression. We further demonstrated that calcitriol inhibited Chk1 phosphorylation at serine 345 induced by PDGF, while having no effect on the phosphorylation of Chk2. Our findings are consistent with those of others that suggest that Chk1 activation promotes cell growth rather than serving as a checkpoint to inhibit mitogenesis (Ren *et al.*, 2002; Maude and Enders, 2005). In MC3T3-E1 cell line, calcitriol was reported to inhibit proliferation by inhibiting Chk1 transcription by localizing E2F.p107/p130 repressor complexes to Chk1 promoter (Verlinden *et al.*, 2007). As Cdc25 phosphatases are substrates for activated Chk1 and mediate delays in progression of mitosis in a Cdc-dependent manner, we examined whether Cdc24C was modulated by calcitriol or PDGF, and found Cdc25 and Cdc phosphorylation to be unaffected. Taken together, our data support the novel hypothesis that Chk1, but not Chk2, phosphorylation is associated with PDGF-induced ASM growth, and calcitriol decreases Chk1 phosphorylation.

Asthma, a disease characterized by airway inflammation and hyper-responsiveness, may promote airway remodelling as shown by increased ASM mass. The consequence of increases in ASM mass may be irreversible airflow obstruction. The precise mechanisms regulating ASM proliferation in diseases characterized by airway inflammation remain unclear. Recent evidence suggests that ASM derived from subjects with asthma may manifest augmented growth *in vitro* (Roth *et al.*, 2004). In the current study, we confirm that cells derived from subjects with asthma proliferate at greater rates than those derived from normal subjects. In addition, ASM derived from subjects with asthma traverses the cell cycle at a greater rate than ASM derived from normals. Despite the augmented growth seen in the asthma ASM, the growth of both asthma and normal ASM cells was inhibited by calcitriol. Although glucocorticoids are widely used in the management of asthma, the effect of steroids in modulating mitogen-induced ASM growth remains unclear. Mitogens such as thrombin, histamine and PDGF elicit their proliferative responses by multiple signalling and transcriptional mechanisms, and the effects of corticosteroids on ASM growth appear controversial (Panettieri, 2004). For instance, corticosteroids reduced thrombin-mediated augmentation of cyclin D1 protein and attenuated phosphorylation of Rb protein, yet have limited efficacy in inhibiting ASM mitogenesis in response to receptor tyrosine kinase-activating mitogens (Stewart *et al.*, 1995; Fernandes *et al.*, 1999; Ammit and Panettieri, 2001). Others report that the effects of corticosteroids are dependent on ASM–matrix interactions (Bonacci *et al.*, 2003). Glucocorticoids in our study had little effect on normal or asthma-derived ASM cells, and, in most cases, augmented mitogen-induced proliferation. In the current study, growth responses mediated by receptor tyrosine kinase pathways (PDGF) and G-protein coupled receptor pathways (thrombin) were examined. The sensitivity of either pathway to calcitriol was comparable in both asthma and normal ASM cells.

Vitamin D plays an important role in calcium and bone homeostasis, but may also modulate airway inflammation and cell growth. Recent studies suggest that alterations in vitamin D metabolism or supplementation with vitamin D may modulate airway inflammation or response to asthma therapy (Banerjee *et al.*, 2008). In the current study, we demonstrated that calcitriol inhibited ASM cell proliferation by preventing progression of the cell cycle and not by inducing apoptosis. The precise signalling mechanisms by which calcitriol inhibited ASM cell growth remain unclear, but involved diminished hyperphosphorylation of Rb protein and the phosphorylation of Chk1 while having no effect on other inhibitors of the cell cycle. Taken together, these data suggest that calcitriol may be a useful therapeutic agent in the prevention of increases in ASM mass described in patients with inflammatory airway diseases, such as asthma and COPD.

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## Conflicts of interest

None.

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